

## Viability and infectivity of *Trichinella spiralis* muscle larvae in frozen horse tissue

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### Abstract

Many aspects of the biology and epidemiology of *Trichinella* infection in the horse are poorly understood, including survival of *Trichinella* spp in horse muscle. In this study, we have assessed the freeze tolerance of *T. spiralis* in horse meat stored at 5, –5, and –18 °C for 1 day to 24 weeks. Results demonstrate a steady reduction in the number of live ML recovered from the cold stored meat samples. On Day 1, recovery of live larvae had been reduced by 18.6%, 50.1%, and 37.2%, and by 4 weeks, recovery of larvae had been reduced by 65.4%, 66.5%, and 96.2% in samples stored at 5, –5, and –18 °C, respectively. Infectivity results (measured as reproductive capacity index (RCI)) from mice inoculated with larvae recovered from non-frozen meat samples at day 0 was 23.5. Following storage at –18 °C for one and two days, the RCIs were 2.09 and 0.99, respectively. Small numbers of infective larvae were still present in meat samples stored at –18 °C for 4 weeks. The RCI of ML recovered from meat samples stored at –5 °C was 14.99 and 6.36 at 2 weeks and 4 weeks respectively; the RCI of samples stored at 5 °C was 23.1 at 8 weeks, and fell rapidly thereafter (12 week RCI 1.33; 0 at 24 weeks). These data demonstrate that infective *T. spiralis*, a non-freeze tolerant species, can survive for at least 4 weeks in horse tissue frozen at –5 or –18 °C, and that the numbers of infective larvae decrease substantially by day 2 at –18 °C and by week 4 at –5 °C.

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### 1. Introduction

Human trichinellosis results from the consumption of raw or undercooked pork, horsemeat, or game meats (e.g., bear, wild boar) containing infective larvae of parasites of the genus *Trichinella*. Considerable effort has gone into protecting consumers of pork from exposure to *T. spiralis* including mandatory carcass

inspection programs for pigs and horses, post-slaughter processing methods for pork ([Federal Register, 9CFR 318.10](#)), and consumer education campaigns describing proper cooking temperatures to render potentially infected meat safe for consumption. Many consumers also understand freezing pork in the home as a method to render meat safe. However, the effectiveness of freezing is influenced by a variety of factors, including the species or genotype of *Trichinella* contained in the meat, and the type of meat (pig, horse, or game animals). Eight sibling species and three genotypes of undetermined taxonomic status have been identified in the genus ([Kapel, 2000](#); [Murrell et al., 2000](#); [La Rosa](#)

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et al., 2003; Pozio and Zarlenga, 2005). Worldwide geographic distribution of these isolates has been described (Pozio et al., 1992; Kapel, 1997; Pozio et al., 1998; Pozio, 2001a,b; Zarlenga et al., 2006). Two of the described sibling species, *T. nativa* and T-6, are capable of surviving for extended periods of time in frozen muscle at temperatures from  $-5$  to  $-18$  °C (Kapel et al., 1999; Malakauskas and Kapel, 2003; Hill et al., 2005), while other species, including *T. spiralis*, are susceptible to freezing. Most studies testing freeze tolerance in *Trichinella* have been conducted in pork. The horse is considered an aberrant host for *Trichinella* spp, however, numerous outbreaks of trichinellosis have occurred in humans resulting from consumption of undercooked horsemeat (Ancelle, 1998; Boireau et al., 2000; Touratier, 2001). Although individual carcass testing for both the import and export markets is generally required, gaps in the inspection process have resulted in continued outbreaks (Ancelle, 1998; Pozio et al., 2001; Webster et al., 2006). Despite the importance of horses as a source of human trichinellosis, many aspects of the biology and epidemiology of *Trichinella* infection in the horse are poorly understood, including survival of *Trichinella* spp. in horse muscle. Kapel et al. (2004) has reported that *T. spiralis* muscle larvae (ML) can survive for 4 weeks in horse muscle frozen at  $-18$  °C, in contrast to the lack of survival seen in *T. spiralis* ML in pork frozen at  $-18$  °C. In this study, we have assessed the freeze tolerance of *T. spiralis* in horse meat stored at 5,  $-5$ , and  $-18$  °C for 1 day to 24 weeks.

## 2. Materials and methods

### 2.1. Source of horses, condition, and vaccinations administered

Twelve adult horses were acquired at a public auction (Fauquier Livestock Exchange, Marshall, VA). Horses were 3–15 years of age; 6 of the horses were mares, 6 were geldings. The details of the horse acquisition and management during the study has been described; this group of 12 horses is the 1 year cohort described in Hill et al., 2007. Horses were vaccinated 10 days after acquisition against rabies, tetanus, influenza (A-1 and A-2), rhinopneumonia, Eastern and Western equine encephalitis, Potomac horse fever, and *Streptococcus equi* (strangles). No anthelmintics were administered to horses prior to parasite inoculation or during the study. Horses were allowed to acclimate on assigned pastures for 1 month prior to parasite infection.

### 2.2. Source of parasites for horse infections

The Beltsville strain of *Trichinella spiralis* (T1) used to inoculate horses was propagated by serial passage in female Sprague-Dawley rats (Taconic Farms, Germantown, NY). The muscle larvae (ML) burden in rat tissues was determined as described by Hill et al. (2007). The mean number of intact ML per gram of blended rat tissue was determined to be 6000 larvae per gram (LPG).

### 2.3. Horse infections and dosage groups

Four horses each were inoculated with 1000 ML, 5000 ML, or 10,000 ML using a weighed amount of the blended rat muscle described above. Individual muscle tissue samples were coated with horse feed (Reliance 10 Textured Feed, Southern States Cooperative, Richmond, VA) to make the samples palatable to the horses, and each sample was presented individually to each horse on a small tray; consumption of the sample was observed to completion.

### 2.4. Horse necropsy

Eleven horses (4 inoculated with 1000 ML, 4 inoculated with 5000 ML, and 3 inoculated with 10,000 ML; one mare inoculated with 10,000 ML died 7 months p.i. due to colic unrelated to the *Trichinella* infection) were humanely euthanized by the Institutional Veterinary Medical Officer at 12 months post-inoculation (p.i.). Horses were euthanized individually by tranquilization (0.01–0.04 mg/kg Butorphanol tartrate administered IV with 0.1–0.5 mg/kg Xylazine) followed by intravenous administration of Buthanasia solution to effect (Pentobarbital sodium at 85.5 mg/kg; NLS Animal Health, Owings Mills, MD).

### 2.5. Digestion of tissues and determination of ML burden

The masseter, diaphragm, supraspinatus, and trapezius were collected unilaterally or bilaterally from each horse at necropsy. Muscles were trimmed of fat and connective tissue, cut into smaller pieces, and the total amount of each muscle was individually ground using Hobart Model 4612 meat grinders (Hobart Corp., Troy, OH). One hundred grams of meat from individually ground samples were digested by the method of Gamble (1996). The digest sediments containing ML were counted on a stereo microscope at 40× magnification, and the LPG was calculated for each individual muscle.

Ground muscle tissues containing 3 to 48 LPG were mixed together using the Hobart grinder to provide a single blended pool of ground muscle tissue with a uniform larval density of 20 LPG. Approximately 2000 live ML were contained in each 100 gm sample of horsemeat used in the study (20 LPG  $\times$  100 gms). On Day 0 of cold storage, 71.6% of ML (three 100 gm samples digested, mean number of live ML recovered = 1433.3) were recovered from un-treated meat samples. This figure was used as the best estimate for recovery of live ML from un-treated meat samples (baseline recovery). Throughout the study, live ML were defined as observed larvae that were actively motile when warmed to 37 °C or exhibited a typical coiled appearance.

### 2.6. Determination of freeze tolerance

One hundred gram samples of the blended muscle containing 20 LPG of *T. spiralis* were packaged in plastic bags (Micro-Seal, Dazey Corp., New Century, KS, USA), and the opening was heat sealed. Three storage temperatures, 5, –5, and –18 °C, were tested, and 3–100 g bags of blended muscle were stored at each temperature for each time period. Freezer temperatures were monitored twice daily to assure maintenance of desired temperatures. Blended muscle was stored at each temperature for 0, 1, and 2 days, 1, 2, 4, 6, 8, 12, and 24 weeks. At the end of each time period for each temperature, the triplicate samples were removed from storage and allowed to thaw for 1–2 h. Samples were then individually digested as described above. Larvae from each sample were suspended in 1 ml of 0.85% saline warmed to 37 °C to increase motility of live larvae. The number of live, motile larvae were then counted, and the number of live larvae per gram of tissue was determined for each sample.

### 2.7. Infectivity of recovered ML for mice

Muscle larvae were collected from the digested muscle described above for each time period beginning at time 0 and each temperature tested, and three Swiss-Webster mice were each orally inoculated with 500 live, motile ML isolated from the treated samples. Only live, motile larvae were enumerated in the inoculum. If the total number of live ML collected from each sample was not sufficient to give each mouse 500 ML, then the total number of larvae was divided into 2 aliquots and inoculated into 2 mice (no more than 500 ML per mouse). After 35 days, mice were skinned, eviscerated and digested as described above to detect ML. The

reproductive capacity index (RCI; number of larvae recovered/number of larvae inoculated) was calculated for ML recovered from inoculated mice. The mean number of ML obtained from mice inoculated with 500 live ML isolated from un-treated meat samples on day 0 of cold storage was 11,760 ML, resulting in a baseline RCI of 23.5.

## 3. Results

Results demonstrated a steady reduction in the number of live, motile ML recovered from the cold stored meat samples (Table 1). By Day 1, recovery of live larvae had been reduced by 18.6%, 50.1%, and 37.2% in samples stored at 5, –5, and –18 °C,

Table 1

Mean, SD of live, motile muscle larvae recovered from triplicate samples stored at 5, –5, and –18 °C

Cold storage time	Storage temperature		
	5 °C	–5 °C	–18 °C
Day 1	1166.6*	714.6	900.0
	208.1**	147.8	300.0
	<b>–18.6%</b> ***	<b>–50.14%</b>	<b>37.2%</b>
Day 2	1233.3	1166.6	833.3
	611.0	378.5	208.1
	<b>–13.9%</b>	<b>–18.6%</b>	<b>–41.8%</b>
Week 1	866.6	600.0	566.6
	404.1	360.5	230.9
	<b>–39.5%</b>	<b>–58.1%</b>	<b>–60.4%</b>
Week 2	861.0	446.6	275.6
	517.3	196.2	62.0
	<b>–39.9%</b>	<b>–68.8%</b>	<b>–80.8%</b>
Week 4	496	479.3	54.6
	54.3	79.3	19.1
	<b>–65.4%</b>	<b>–66.5%</b>	<b>–96.2%</b>
Week 6	365	94	107
	170	161	186
	<b>–74.5%</b>	<b>–93.4%</b>	<b>–92.5%</b>
Week 8	154.3	0	0
	137.5	0	0
	<b>–89.2%</b>	<b>–100%</b>	<b>–100%</b>
Week 12	215	0	0
	45.8	0	0
	<b>–84.9%</b>	<b>–100%</b>	<b>–100%</b>
Week 24	0	0	0
	0	0	0
	<b>–100%</b>	<b>–100%</b>	<b>–100%</b>

Time 0 mean = 1433.3, S.D. 152.7.

\* Mean number of live, motile muscle larvae recovered from triplicate samples.

\*\* standard deviation.

\*\*\* Percent reduction from Time 0 (in bold).

Table 2

Infectivity of live ML recovered from infected horsemeat as determined by oral inoculation into mice

Cold storage time <sup>a</sup>	Mean ML recovery from 3 mice/RCI		
	5 °C	−5 °C	−18 °C
Day 1	11,283/ <b>22.56</b> **	12,642/ <b>25.28</b>	1045/ <b>2.09</b>
Day 2	11,180/ <b>22.36</b>	10,010/ <b>20.02</b>	496/ <b>0.99</b>
Week 1	11,840/ <b>23.68</b>	6466/ <b>12.93</b>	33/ <b>0.06</b>
Week 2	12,066/ <b>24.13</b>	7496/ <b>14.99</b>	5/ <b>0.01</b>
Week 4	10,235/ <b>20.47</b>	3180/ <b>6.36</b>	306/ <b>0.61</b>
Week 6	4019/ <b>8.03</b>	3/ <b>0</b>	0/ <b>0</b>
Week 8	1286/ <b>2.57</b>	0/ <b>0</b>	0/ <b>0</b>
Week 12	444/ <b>0.88</b>	0/ <b>0</b>	0/ <b>0</b>
Week 24	0/ <b>0</b>	0/ <b>0</b>	0/ <b>0</b>

RCI shown in bold.

<sup>a</sup> Time 0 infectivity based on a dose of 500 live ML per mouse; mean recovery ( $n = 3$  mice) RCI 23.5; 11,760 ML.

\* Mean number of larvae recovered from 3 mice.

\*\* RCI calculated as follows: Mean number of larvae recovered from mice/number of live larvae inoculated into mice.

respectively. By 4 weeks, recovery of live larvae had been reduced by 65.4%, 66.5%, and 96.2% in samples stored at 5, −5, and −18 °C, respectively. No live ML were recovered from samples stored at −5 °C or −18 °C after 6 weeks; live ML were recovered from samples stored at 5 °C until week 12.

Of interest are the infectivity results from mice inoculated with larvae recovered from cold stored meat samples (Table 2). The RCI in mice inoculated with live ML recovered from samples stored for 1 day at 5 and −5 °C was 22.56 and 25.28, respectively. However, the RCI had been reduced to 2.09 in mice inoculated with live ML recovered from samples stored for 1 day at −18 °C. The infectivity of live ML recovered from meat samples stored at −18 °C for 2 days was further reduced to an RCI of 0.99. Infectivity of live ML recovered from meat samples stored at 5 °C remained high for at least 4 weeks (RCI 20.47), and fell rapidly thereafter. Muscle larvae in meat stored at −5 °C retained infectivity in the mouse for 2 weeks (RCI 14.99), however, by week 4, the RCI had fallen to 6.36, and by week 6 the RCI was 0.

#### 4. Discussion

Though traditionally thought of as a parasite of pigs and carnivorous game animals, *Trichinella* spp. are now recognized as a serious food safety risks to consumers of fresh horsemeat, which is typically eaten raw or undercooked. Although this horse-*Trichinella* association was first described in 1975, relatively little is known of the biology, epidemiology, and persistence of *Trichinella* in the horse. Natural and experimental

infections have been described (Smith and Snowdon, 1987; Soule et al., 1989, 1993; Pozio et al., 1998, 1999), however, the ability of non-freeze tolerant species of *Trichinella* to survive for extended periods in frozen horse tissue has only recently been described (Kapel et al., 2004). In that study, *T. spiralis* ML in pork were killed by 1 week of storage at −18 °C, but survived for 4 weeks at −5 °C. In horsemeat, *T. spiralis* survived for at least 8 weeks at −18 °C; infectivity of these recovered ML was not reported. Previous studies by Malakauskas and Kapel (2003) suggest that the age of the encapsulated larvae may impact its ability to survive in frozen muscle tissue; *Trichinella* ML in rat tissue 10 to 20 weeks post infection were more likely to survive freezing than ML 5 weeks or 40 weeks post infection. In the present study, we confirm that *T. spiralis* (12 months post infection) can persist for extended periods of time in frozen horsemeat. Though motile larvae can be recovered from these frozen tissues, the infectivity of ML recovered from samples stored at −5 and −18 °C is low in mice, and this reduced infectivity occurs rapidly (1 day of storage at −18 °C). Though infectivity of recovered larvae is low in mice, the infectivity of these larvae in humans is currently unknown, as is the ability of this level of infectivity to cause clinical disease in human subjects. Nevertheless, these data are in agreement with a previous observation showing that infectious *T. spiralis* larvae are present in horsemeat following 4 weeks of storage at −18 °C. Whether these data hold true under field conditions is unknown, but a documented risk exists that indicates cold treatment strategies currently utilized for pork should not be applied to horsemeat to prevent trichinellosis. Commercial processing requirements dictated for the treatment of fresh pork products detail specific time and temperature combinations to eliminate the risk of infection with *T. spiralis* to consumers (Gamble et al., 2000). Freezing has also been promoted to consumers of fresh pork as a method to assure safety with respect to possible exposure to *Trichinella* in infected meat. These regulations and recommendations regarding freezing are based on research conducted with *T. spiralis* in pork and should only be applied to that combination of parasite species and host.

#### 5. Conclusions

These data demonstrate that cold treatment strategies currently utilized for pork cannot be applied to horsemeat as a mitigation strategy for control of *Trichinella*. Additional studies are needed to thoroughly define freezing parameters necessary for treatment of

horsemeat in order to render meat safe for human consumption. In those countries where horsemeat is eaten by humans, consumers should be alerted to the risk of acquiring trichinellosis and the importance of thoroughly cooking meat or obtaining assurance that adequate inspection has been performed using a validated method of artificial digestion.

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